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Inhibitory effects of epigallocatechin gallate (EGCG) combined with zinc sulfate and silver nanoparticles on avian influenza A virus subtype H5N1

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Abstract. – OBJECTIVE: Even though antiviral drugs against H5N1 flu infection are accessible, they are still limited by antiviral drug resistance and unfavorable side effects. Thus, this work tested the action of epigallocatechin gallate (EGCG) co-administered with both zinc (II) ions and silver nanoparticles (AgNPs).

MATERIALS AND METHODS: EGCG was used with both zinc sulfate (zinc II) and silver nanoparticles to test their antiviral activities against avian flu subtype H5N1 in embryonated SPF eggs. The MTS test was used to determine the cytotoxicity.

RESULTS: Zinc sulfate (1.5 mg/mL) and silver nanoparticles showed comparable potentiated antiviral action with EGCG (50 μM) against the H5N1 avian flu virus. They decreased the log titer infection by up to 5.7 and 5.6 fold separately with critical antiviral activity ($p<0.01$). In most cases, an illustrative relationship was seen when H5N1 was tested with EGCG and various concentrations of zinc sulfate. The EGCG-AgNPs with zinc sulfate were observed to have very strong antiviral activity ($p<0.001$) against the H5N1 avian influenza virus with a reduction in the log titer of the virus by up to 7.6 times. No cytotoxicity was recognized.

CONCLUSIONS: The potentiated antiviral activity of EGCG by co-administering it with zinc II and AgNPs indicates potential as a multi-activity novel topical therapeutic agent against H5N1 flue. This mix makes the adaptation of the virus difficult, which helps to reduce infection resistance.

Key Words:
Avian influenza A virus subtype H5N1, Epigallocatechin gallate (EGCG), Zinc sulfate, Silver nanoparticles, Antiviral.

Introduction

Recently, a number of spreading viral outbreaks imposed significant burdens on medical services, prompting worldwide concern[1]. Examples of serious outbreaks include the severe acute respiratory syndrome coronavirus (SARS-CoV) in 2002[2], the H1N1 flu pandemic in 2009[3], the H5N1 and H5N7 pandemics of influenza A virus[4], and the disastrous Ebola and Zika epidemics of 2014 and 2015[5]. Among these, H5N1 avian flu has attracted much consideration because of its quick spread around the world and high pathogenicity[6]. The main cause of death in infected patients is diffuse alveolar harm and discharge in the lungs, which are brought about by overactive inflammatory responses[7]. Overproduction of inflammatory cytokines in H5N1-infected mice and humans is called a cytokine storm, which has been recognized as the fundamental driver of death related to this virus[8,9].

Green tea is a popular beverage, and numerous reports show that it has medical advantages, including malignant growth prevention[10,11]. Epigallocatechin gallate or epigallocatechin-3-gallate (EGCG) is a significant ingredient in green tea[10,12]. EGCG and green tea have demonstrated a wide range of antiviral activity against adenovirus, influenza, Zika virus, herpes virus, and hepatitis virus[13-15]. Zinc also has direct antiviral properties (for example, on the flu virus) and antiviral activity when co-administered with plants[16]. A few examinations have also shown intense antiviral activity of AgNPs against different human pathogenic infections, such as respiratory syncytial virus (RSV), influenza, norovirus, hepatitis B virus (HBV), and human immunodeficiency virus (HIV)[17].

Immunization remains the best preventive measure against flu infections. However, inoculation viability is diminishing because influenza infections have a high transformation rate, requiring adjustments in the immuni-

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zation composition, but vaccine creation is always time-consuming[18,19]. Some antiviral drugs, such as zanamivir (Relenza) and oseltamivir (Tamiflu), are being used right now, but the expanding development of drug-resistant strains is influencing their clinical application, and the antiviral medications are not free of adverse side effects[20,21]. Therefore, novel wide-range prophylactic and therapeutic agents are greatly needed for use against H5N1 avian flu. Therefore, we inspected the potentiated antiviral combinations of EGCG, Zn (II), EGCG conjugated with zinc (II) particles, EGCG formed with silver nanoparticles (EGCG-AgNPs), and EGCG-AgNPs co-regulated with zinc (II) particles.

Materials and Methods

Reagents and Virus

(−)-Epigallocatechin gallate (EGCG) (purity ≥ 95%) was purchased from Sigma-Aldrich (Saint Louis, MO, USA), silver nitrate (AgNO3) was purchased from Merck (Darmstadt, Germany), and sodium borohydride (NaBH4) (powder, ≥98.0%) was purchased from Sigma-Aldrich (Saint Louis, MO). Influenza A (H5N1) viruses isolated from birds between 2007 and 2012 were identified as influenza A subtype H5N1 at the Jordan Bio-Industries Center (JOVAC), Amman, Jordan, by using the World Health Organization (WHO) protocol. Virus handling was conducted under enhanced Biosafety Level 3 containment according to institutional guidelines.

Synthesis of EGCG Silver Nanoparticles (EGCG-AgNPs)

EGCG conjugated with silver nanoparticles (EGCG-AgNPs) was synthesized. Briefly, 2 mL of varying concentrations of EGCG solution (25 µM, 50 µM, 100 µM, 150 µM, and 200 µM) were mixed with 2 mL (0.1 mM) of an aqueous silver nitrate solution, and the reaction mixture was magnetically stirred for 20 minutes. Next, 20 µL of freshly prepared 5 mM $NaBH_4$ solution was added to the reaction mixture under stirring. The color of the solution turned from transparent to an earthy yellow color, indicating the decrease of silver particles and the development of EGCG-AgNPs[22,23]. The bioreduction of silver ions to AgNPs was monitored with a UV-2450 double-beam spectrophotometer (Shimadzu, Tokyo, Japan).

Virus Propagation as Control

The working seed of the influenza A subtype H5N1 viruses containing a titer of an egg infectious dose (EID_{50})/mL of 10^8 was diluted in 1 mL of sterile PBS at pH 7.2 (Gibco, Thermo Fisher Scientific, USA). It was then diluted serially using sterile PBS (pH 7.2) until reaching the desired dilution (10^5 EID_{50}/mL). About 0.1 mL of dilution was inoculated into each allantoic hole of 10-day-old embryonated SPF eggs. The eggs were then hatched at 37°C. Eggs containing embryos that died within 24 hours were discarded.

Allantoic liquids (AFs) were collected upon the death of the undeveloped organism or at 72 hours post-inoculation. The infected eggs were chilled at 4°C prior to being harvested. The highest points of the eggs were removed, and the allantoic liquids were gathered by suction. The yolk material and albumin were avoided. All liquids were put away promptly at 4°C and tested for both bacterial contamination and titration[24].

Virucidal Reduction Assay

The working seed of H5N1 flu virus containing the titer of 10^8 EID_{50}/mL was diluted in 1 mL of sterile PBS (pH 7.2) and cultured at 37°C with EGCG at various concentrations for 1 hour to survey the antiviral activity as a log decrease. The virus treated with EGCG was serially diluted to 10^5 EID_{50}/mL, and then 0.1 mL of this dilution was inoculated into the allantoic cavities of 10-day-old embryonated SPF eggs. These were then incubated at 37°C. Eggs containing embryos that died within 24 hours were discarded.

AFs were harvested upon the death of the embryo or at 72 hours post-inoculation. The infected eggs were chilled at 4°C before being harvested. The tops of the eggs were removed, and the allantoic fluids were collected by suction. From that point forward, the titer of the flu infections was monitored for EID_{50}/mL values. EID_{50}/mL was determined and compared with that of the control (0.1 mL 10^5 EID_{50}/mL). Furthermore, 0.1 mL of different antiviral compounds was tested to see the impact on embryonated SPF eggs[24].

Titration of Influenza Viruses (EID_{50}/mL)

The presence of the virus was confirmed by HA, and titration was performed. Viral EID_{50} titers were found by infusing 100 µL of 10-fold dilutions of the virus into the allantoic cavities of 10-day-old eggs. For each dilution, five eggs were used for the exact estimation of the titer. The

50% endpoints were calculated according to the method of Reed and Muench[25] for 50% EID_{50} and are expressed AS log10 EID_{50}/mL[12].

Cytotoxicity

Test solutions were analyzed for cytotoxicity using the Cell Titer 96 Aqueous assay strategy (Promega, Southampton, UK). This was done in a 96-well-plate with a known concentration of Vero cells in each well. 2.5 × 103 cells were used for every well for each plate prepared by the trypsinization of 80% intersecting Vero cells in a T75 flask (Greiner Bio-One, Stonehouse, UK). New reconstituted DMEM cells were cultivated in a 96-well-plate and cultured for 24 hours at 37°C under 5% CO_2. The medium was taken out by aspiration, the cells were washed multiple times with phosphate-buffered saline and quickly supplanted with DMEM media containing the ideal concentrations of test materials.

Cells were cultured for 6, 24, 48, and 72 hours. Cell Titer 96 Aqueous solution was used to identify cell viability and was added to each well. The plates were kept for 1 hour at 37°C under 5% CO_2. The optical thickness was then found at 492 nm with the blank deducted from each sample reading and the mean thickness for the control cells.

Results

Inhibitory Effects of EGCG on Avian Influenza Virus (H5N1)

Embryonated SPF eggs were used for influenza virus propagation with a quantity feasible for vaccine production. The virus log titers were determined at concentrations of 25 μM, 50 μM, 60 μM, 100 μM, 150 μM, and 200 μM. The H5N1 virus' $logEID_{50}$/mL value at 60 μM EGCG was reduced by 4 times. Increasing the concentration of EGCG to more than 60 μM did not produce a greater magnitude of the effect (Figure 1). Therefore, in subsequent assays, the 60-μM concentration of EGCG was used.

Virucidal Effect of EGCG when Combined with Different Levels of Zinc Sulfate

Increasing levels of zinc sulfate were associated with increased antiviral effect and decreased value of $logEID_{50}$/mL of the H5N1 virus. We observed a virucidal log reduction when we combined 60 μM of EGCG with different concentrations of zinc sulfate (Zn II). The reduction of

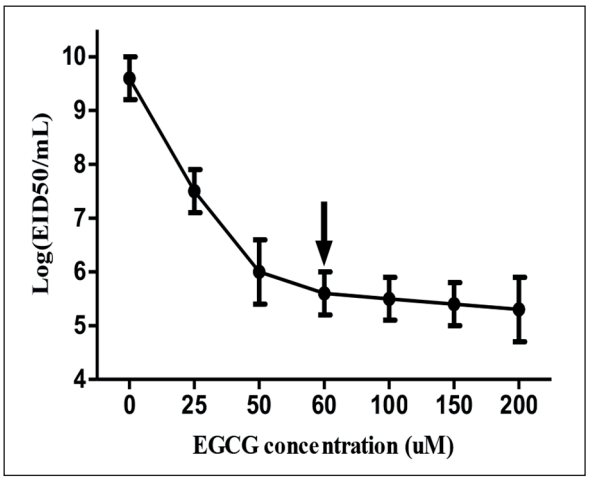

Figure 1. Inhibitory effects for different concentrations of EGCG on avian influenza virus A (H5N1) replication in SPF eggs in EID50/mL test.

$logEID_{50}$/mL for the virus when treated with 60 μM of EGCG and when combined with 1.5 mg/mL of zinc sulfate (Zn II) were 5.6±0.4 times to 4.2±0.4 times, respectively, with a maximal reduction in $logEID_{50}$/mL of 1.4 times when they were compared together (Figure 2). Also, in Figure 2, increasing the concentration of zinc sulfate by more than 1.5 mg/mL did not produce a greater magnitude of the effect.

Virucidal Effect of zinc Sulfate, EGCG, EGCG with Zinc Sulfate, EGCG-AgNPs, and EGCG-AgNPs with Zinc Sulfate

The $logEID_{50}$/mL value of the viruses in SPF eggs was 9.6 in the control group. With

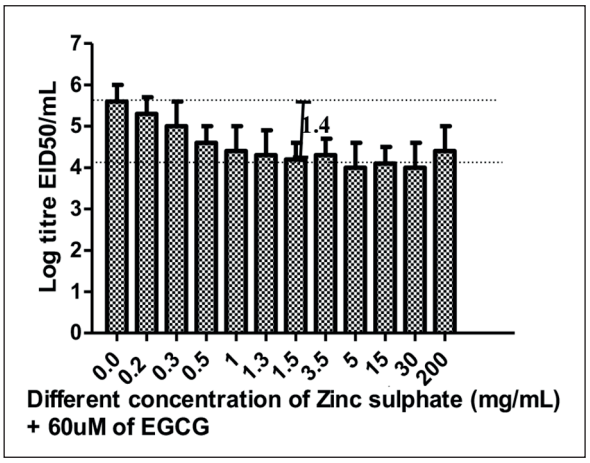

Figure 2. The effect of zinc sulfate concentration (mg/mL) on the potentiated antiviral activity of EGCG (60 μM) with the maximum antiviral potentiated activity at 1.5 mg/mL.

1.5 mg/ml of zinc sulfate, the virus log titer was 9±0.6 without significant antiviral activity (p>0.05). With 60 μM of EGCG, logEID_{50}/mL was 5.6±0.4 with significant antiviral activity (p<0.05). When 60 μM of EGCG and 1.5 mg/mL of zinc sulfate were combined, logEID_{50}/mL was 4.2±0.4 with highly significant antiviral activity (p<0.01).

With EGCG-AgNPs, logEID_{50}/mL was 4±0.4 with highly significant antiviral activity (p<0.01). When the EGCG-AgNPs and 1.5 mg/mL of zinc sulfate were combined, logEID_{50}/mL was 2.5±0.6. The virucidal log reduction effect was 7.1 compared with the control with very strong significant antiviral activity (p<0.001), as shown in Figure 3.

Cytotoxicity

The cytotoxicity of 60 μM of EGCG, 1.5 mg/mL of zinc sulfate, the combination of 60 μM of EGCG + 1.5 mg/mL of zinc sulfate, 60 μM of EGCG-AgNPs, and combination of 60 μM of EGCG-AgNPs + 1.5 mg/mL of zinc sulfate in cell culture were evaluated using an MTS proliferation assay. There was no huge distinction (p>0.05) between the applied formulations any time over 72 hours, and there was no lessening in the level of viable cells in comparison with the control. Accordingly, no cytotoxic impact was seen among the entirety of the compounds at the concentrations used in this work (Figure 4).

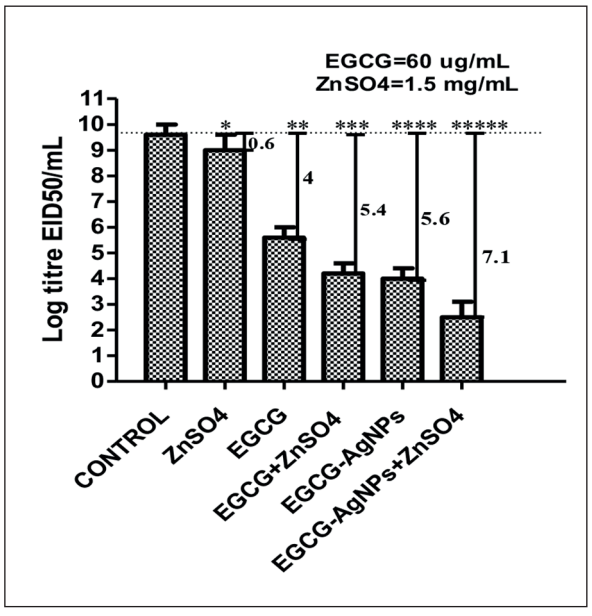

Figure 3. The log EID50/mL of avian influenza A virus subtype H5N1; H5N1 virus alone as control and also with zinc sulfate, EGCG, EGCG + zinc sulfate, EGCG-AgNPs, and EGCG-AgNPs + zinc sulfate. (*p>0.05, **p<0.05, ***p<0.01, ****p<0.01, *****p<0.001).

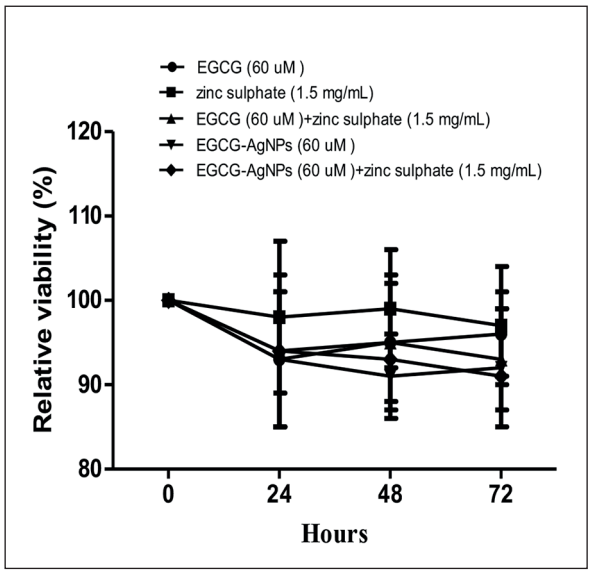

Figure 4. Cytotoxicity effect of (1) EGCG (60 μM), (2) zinc sulfate (1.5 mg/mL), (3) the combination of EGCG (60 μM) + zinc sulfate (1.5 mg/mL), (4) EGCG-AgNPs (60 μM), and (5) the combination of EGCG-AgNPs + zinc sulfate on virus cells. No cytotoxic effect was observed among all of the compounds at the concentrations used in this study.

Discussion

The antiviral activity of EGCG against the influenza virus was reported for the first time in 1993[26]. In the current study, at the concentration of 60 μM of EGCG, a significant decrease (p<0.05) in the log titer of the virus was observed. EGCG appears most likely to inhibit the early stages of infection, such as attachment, entry, and membrane fusion, by interfering with viral membrane proteins. Also, an indirect effect on host cell might affect the fusion with the cell membrane of the virus[15,27].

The explanation of these results is that the ability of EGCG to bind with the surface of the viral envelope deforms the phospholipids, thus inducing the destruction of the virion. In addition to its virucidal capacity, some studies[28,29] have shown that EGCG can act by blocking viral attachment receptors of the target cell. This is in accordance with several studies[6,10,29] that prove that EGCG has the ability to inhibit different

subtypes of influenza virus' growth, including A/H1N1, A/H3N2, and B viruses, which currently infect humans.

Although zinc II possesses direct antiviral properties against viruses[16], we noticed a slight decrease in the titer of the virus at 1.5 mg/mL of zinc sulfate with no significant reduction ($p>0.05$). Despite the limited antiviral activity of zinc sulfate, the virus treated with 60 μM of EGCG combined with 1.5 mg/mL of zinc sulfate had a highly significant reduction in the titer ($p<0.01$), which indicates the occurrence of a dual effect. This increases the possibility that the antiviral activity produced direct interaction of EGCG with the viral envelope[28]. In zinc sulfate, the Zn^{2+} ions may first allow for the EGCG to combine with the glycoprotein envelope, thus destroying it. The zinc may also block the protease activity and polymerase enzymatic processes, as well as physical processes such as virus attachment, infection, and uncoating[16].

In addition, a highly significant reduction in the titer of influenza virus ($p<0.01$) in EGCG-AgNPs was noticed in this study. The silver nanoparticles can be an effective antiviral agent against HSV-1, HAV-10, CoxB4[30], and influenza virus[31]. Although the mechanisms of AgNPs have not been determined, the size of the silver nanoparticles (1-10 nm) allows them to bind to the viral envelope glycoproteins, thereby preventing the viral entry into the host cell[32].

A very strong inhibitory effect ($p<0.001$) in preventing the replication of the virus was observed for the EGCG-AgNPs with zinc sulfate. This raised the possibility that a triple effect was exerted on not only the initial infection by the viruses but also other steps of the infectious cycle. In addition, the EGCG possesses inhibition activity for both hemagglutination and neuraminidase[29].

Despite EGCG having been shown to have natural antiviral activity, some works have observed that EGCG at a higher concentration exhibits cytotoxicity to various cell lines[33]. The EGCG showed some toxicity after 72 hours in reducing Vero E6 cell viability by 20%[13]. However, no cytotoxic effect was observed among EGCG, ZnSO4, EGCG-AgNPs, EGCG with ZnSO4, and EGCG-AgNPs with ZnSO4 at the concentrations used in this study.

By extrapolation, the effective use of EGCG or EGCG-AgNPs with $ZnSO_4$ may be required to bring about a virucidal effect outside of the host cell post-growth and help dispose of the viral vesicles. This would forestall the transmission of the infection by various routes, such as mouth to mouth. Also, EGCG would hinder replication of a virus inside neighboring cells, forestalling the development of a viral group, which can forestall a cold-sore outbreak. Additionally, an advantage of the therapeutic combination is that the antiviral action incorporates a potentiated interaction between zinc particles and the complex combination of phytochemicals or nanotechnology in phytochemicals, and such potentiation is accepted to restrain microbial resistance by making adaptation for microbes difficult[34]. A mutation in a virus that confers resistance does not affect the efficacy of silver nanoparticles[35].

Conclusions

This is the first study demonstrating that EGCG combined with zinc II and AgNPs may have a good possibility to be used in the therapy and prevention of infections because it inhibits the H5N1 influenza virus' entry into the host cell *in vitro*. The mechanism is probably related to the destruction of the virus particle and inhibition of protease, polymerase, hemagglutination, and neuraminidase activity. The potentiated antiviral activity of EGCG co-administered with zinc II and AgNPs has a potential in a novel multi-activity topical therapeutic agent against the H5N1 avian flu virus. This mix makes the adaptation of the virus difficult, which helps limit infection resistance and is not affected by virus mutations.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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